DERIVATISED ANTIBODIES WITH EXPOSED CARBOHYDRATE CHAINS CAPABLE OF BINDING TO IMMOBILISED IGG

Field of the Invention

The present invention relates to derivatised antibodies and their use in the diagnosis and treatment of

5   conditions associated with immobilised IgG, specifically autoantibodies, and in particular to conditions associated with elevated levels of immobilised IgG, such as rheumatoid arthritis (RA). The present invention also relates to a method of producing the derivatised

10   antibodies.

Background of the Invention

A central problem in clinical rheumatology is the need to define at presentation using non invasive techniques

15   those patients who will go on to develop erosive joint disease. If such patients can be identified, then aggressive therapy can be targeted to them and more conservative therapy can be reserved for the remaining patients with RA who do not go on to erosive joint

20   disease. This would improve the treatment of both groups, since early treatment in the erosive group can prevent the progression of disease and in the benign group reduce morbidity due to less frequent and severe drug reactions and toxicity.

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A number of clinical parameters have been assessed for their ability to predict outcome of erosive joint disease. These include age, sex, RF status, genetic background (HLA) and serum agalactosyl IgG levels (see

30   for example JP-A-5087814). However, prior studies have found that no single parameter is useful as a predictor. However, a combination of agalactosyl IgG levels, age of

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onset, gender, functional assessments and RF titre
predicts the course of RA correctly in 94% of patients.

Agalactosyl IgG is an IgG glycoform which is increased in
patients with rheumatoid arthritis, tuberculosis, Crohn's
disease, juvenile onset arthritis, sarcoidosis, type I
insulin dependent diabetes, type II diabetes and a form
of leprosy called erythema nodosum leprosum. Agalactosyl
IgG is thought to cause pathology in the respective
target tissues by a variety of mechanisms including:

(a)   Tissue damage via the interaction of *in situ*
immobilised agalactosyl IgG and the lectins mannose
binding protein (MBP), tumour necrosis factor (TNFα) and
transforming growth factor (TGFβ). The tissue
immobilised autoantibodies can bind these inflammatory
mediators and trap them in the tissue, leading to tissue
damage.

(b)   Agalactosyl IgG is thought to cause systemic
pathology by forming self-associating immune complexes.

The target organ or tissue for agalactosyl IgG action
depends on the specificity of the autoantibody. In the
case of rheumatoid arthritis, it is thought that
autoantibodies against type II collagen are targeted to
the joint. Cross-sectional studies of rheumatoid
arthritis patients have demonstrated a frequency of only
30% for patients with detectable antibodies to type II
collagen late in disease. In early RA the frequency may
reach 70%. The problem of identifying patients likely to

develop erosive joint disease is complicated as three
groups of rheumatoid arthritis patients can be classified
by their anti-collagen autoantibody serum titres.

5   Group 1:   Normal titre of anti-type II collagen
antibodies.

Group 2:   Elevated titre of anti-type II collagen
antibodies which remain constant.

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Group 3:   Elevated titre of anti-type II collagen
antibodies early in disease with a decrease to normal
level later in disease.   In this case, the decrease in
the level of serum antibodies to collagen may include:

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(a)   Intrasynovial synthesis of antibodies to collagen
(in contrast to systemic production);

(b)   Removal of antibodies to collagen secondary to
20   immune-complex formation; and/or,

(c)   Serum skimming by immobilisation of antibodies to
damaged cartilage surfaces.

25   Of the three groups described above, Group 3 will go on
to get severe erosive disease and Group 1 mild disease.
Therefore, serum antibody levels cannot discriminate
between Group 1 and 3 once the levels of antibody in
Group 3 has returned to normal.

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While, the affected synovial joints of Group 3 patients

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(in contrast to Group 1) are coated with anti-type II
collagen antibodies of the agalactosyl IgG glycoform,
joint biopsy is not a routine nor desirable procedure to
detect these antibodies.

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Summary of the Invention

Broadly, the present invention provides derivatised
antibodies, the antibodies being derivatised to expose
carbohydrate chains so that the antibodies are capable of
10   binding to immobilised IgG, such as agalactosyl IgG.
Thus, the derivatised antibodies can be employed in the
diagnosis and treatment of conditions associated with
immobilised IgG, such as rheumatoid arthritis (RA) (Group
3 versus Group 1 above). In particular, the present
15   invention provides a non-invasive technique which can
detect the immobilised IgG *in situ*, thereby assisting in
the diagnosis or treatment of conditions associated with
it. The present invention can help to avoid measuring
the range of clinical parameters currently used to
20   determine the likely course of RA.

Accordingly, in a first aspect, the present invention
provides a derivatised antibody, the antibody being
derivatised to expose carbohydrate chains so that the
25   antibody is capable of binding to immobilised IgG, e.g.
agalactosyl IgG. Preferably, the derivatised antibodies
are also capable of self associating or "stacking" at the
site of the immobilised IgG to assist in diagnosis or
therapy of the patient by amplifying the concentration of
30   the derivatised antibody that can be delivered to the
site of the immobilised IgG. The present invention

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further provides the above antibodies for use in a method
of medical treatment.

The present invention is applicable to the diagnosis or

5   treatment of conditions associated with immobilised IgG,
and in particular agalactosyl IgG, such as autoimmune
disorders including rheumatoid arthritis, juvenile
arthritis, Crohn's disease, type I insulin dependent
diabetes, type II diabetes, sarcoidosis, erythema nodosum

10   leprosum and tuberculosis. In a preferred embodiment,
the present invention can be employed in the diagnosis of
erosive joint disease by detecting the presence of tissue
immobilised anti-type II collagen antibodies produced in
rheumatoid arthritis patients susceptible to erosive

15   joint disease (Group 3).

In a further aspect, the present invention provides a
method of producing a derivatised antibody, the method
comprising treating a precursor antibody to expose

20   carbohydrate chains so that the antibody is capable of
binding to immobilised IgG. Preferably, the method
involves thiolating the antibody in the presence of
carbonate, such as ammonium carbonate or sodium
carbonate. In some embodiments of the invention, only a

25   fraction of the total derivatised antibodies can self
associate or stack, referred to as the "imaging fraction"
below. In this case, preferably the method additionally
involves the step of separating this fraction of the
derivatised antibodies, e.g. using Con A chromatography

30   or by pH elution of the derivatised antibody from a
Protein A affinity column.   In a preferred embodiment,

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the precursor antibody has Fc carbohydrate chains which
terminate with N-acetylglucosamine (GlcNAc) residues.
The proportion of such chains on the precursor antibody
can be increased from naturally occurring levels by the

5   use of β-galactosidase to remove terminal galactose
residues. The imaging fraction of the derivatised
antibodies should also contain Fab associated
carbohydrate chains.

10   The method flips the internal carbohydrate chains, e.g.
Fc carbohydrate chains, from their binding sites on the
interstitial protein surface. The IgG is then chemically
derivatised in a manner which prevents the carbohydrates
from returning to their interstitial positions.

15   The binding between the immobilised IgG and the
derivatised antibodies can be via the Fab carbohydrate
chains of the derivatised antibodies binding to Fc
binding sites or surfaces of the immobilised IgG and/or

20   exposed Fab carbohydrate chains of the immobilised
antibodies binding to Fc binding sites or surfaces
exposed on the derivatised antibodies by the
derivatisation reaction. Thus, in either case, the
binding reaction is between a carbohydrate chain and a

25   carbohydrate binding site on the antibody and is not
linked to the immunological binding domain of the
antibodies. In the case of agalactosyl IgG, as these
antibodies lack galactosyl and sialic acid residues, this
provides a binding site that can receive the available

30   Fab carbohydrate chains of the derivatised antibodies.
In preferred embodiment, the derivatised antibodies can

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bind to themselves as well as binding with the immobilised IgG. This can happen if the flipping out of the carbohydrate chains opens up a binding site that is capable of binding carbohydrate chains on other antibodies (Fab, Fc etc). This stacking of the antibodies can provide a method of delivering the functional moiety to the locations in a patient where immobilised IgG concentrates, and also a method of concentrating the derivatised antibodies at these locations.

The precursor antibody can be polyclonal or monoclonal, and can be IgE, IgA, IgM (monomer), IgD or IgG. The production and purification of antibodies is described in detail below. In the examples below, the precursor antibody used is Sandoglobulin or CLB, polyclonal IgG mixtures.

In a further aspect, the present invention provides the use of a derivatised antibody for the preparation of a medicament for the treatment or diagnosis of a condition associated with immobilised IgG, the antibody being derivatised to expose carbohydrate chains so that the antibody is capable of binding to immobilised IgG.

In some embodiments, the derivatised antibody can be conjugated to a functional moiety, such as a label, toxin, drug, prodrug, effector or other moiety. Labels allow the antibody to be visualised *in vivo* or *in vitro*, indicating the presence and/or location of agalactosyl IgG. In a preferred embodiment, the label is a

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radioactive label such as ^{99m}Tc. Where the functional
moiety is a drug, prodrug or effector, the derivatised
antibodies can deliver the functional moiety to locations
in a patient's body where immobilised IgG is found and
where the action of the drug, prodrug or effector is
required.

Alternatively or additionally, the precursor antibody can
itself have a therapeutic effect when delivered to
locations in which immobilised IgG accumulates. By way
of example, a derivatised antibody with specificity
against an inflammatory mediator such as TNF-α could be
used to ameliorate the effects of these substances at
locations in a patient having elevated levels of
immobilised IgG. In a further aspect, the present
invention provides a method of diagnosing *in vivo* a
condition associated with immobilised IgG, the method
comprising (a) exposing a patient to derivatised
antibodies, the antibodies being derivatised to expose
carbohydrate chains so that the antibodies can bind to
immobilised IgG and being labelled, and (b) using the
label to detect the presence of the immobilised IgG.
Conveniently, the derivatised antibodies can be
administered to the patient by injection.

In a further aspect, the present invention provides a
pharmaceutical composition comprising one or more of the
above mentioned antibodies in combination with a
pharmaceutically acceptable carrier.

The present invention will now be described by way of

example and not limitation with referenc to the
accompanying figures.

Brief Description of the Drawings

5   Figure 1 shows the anti-GlcNAc reactivity of immobilised
     Sandoglobulin or derivatised Sandoglobulin (HIG).
     Solutions containing various concentration of
     Sandoglobulin or HIG were added to microtitre wells
     coated with protein A. The top figure shows the anti-
10   GlcNAc reactivity of the immobilised IgG or HIG after
     heating the plate 85°C for 10 minutes to mildly denature
     the protein and expose the carbohydrate residues. The
     bottom figure shows the anti-GlcNAc reactivity without
     the heating step.

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     Figure 2 shows the anti-GlcNAc reactivity of immobilised
     Sandoglobulin (top) or HIG (bottom). Solutions
     containing various concentrations of Sandoglobulin of HIG
     were added to micro titre plates directly/or to plates
20   coated with protein A. Prior to the addition of the
     anti-GlcNAc monoclonal antibody the protein A coated
     plates were either heated to 85°C for 10 minutes or
     incubate at room temperature for a similar period.

25   Figure 3 shows the results of applying Sandoglobulin or
     HIG (4852) to an affinity column containing immobilised
     Concanavalin A. Three fractions were obtained: unbound,
     weakly bound - eluted with 100 MM α-methyl mannoside -
     and strongly bound - eluted with 100 mM HCl. Various
30   concentrations of each of the three fractions were
     incubated in protein A coated microtitre wells. The

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anti-GlcNAc reactivity of the immobilised Sandoglobulin
of HIG fractions were measured. The bottom figure show
the percentage of unbound and bound material for three
different HIG preparations and underivatised

5   Sandoglobulin.

Figure 4 shows anti-GlcNAc reactivity of immobilised CLB
and derivatised CLB (HIG-62759) and Sandoglobulin and
derivatised Sandoglobulin (HIG 4855). Solutions

10 containing various concentrations of the different
preparations were added to micro titre plates coated with
protein A. The plates were then heated at 85°C for 10
minutes.

15 Figure 5 shows the anti-GlcNAc reactivity of immobilised
Sandoglobulin derivatised with iminothiolane using
different buffers. Solutions containing various
concentrations of derivatised IgG were added to
microtitre plates coated with protein A and then heated

20 at 85°C for 10 minutes.

Figure 6(a) shows how the derivatised antibodies of the
invention bind to anti-type II collagen IgG antibodies
that are found immobilised in the joint of RA patients.

25 Figure 6(b) shows the difference in the structure of
carbohydrate chains in agalactosyl and normal antibodies

Figure 7 shows a schematic drawing of the species
involved in the experiments shown in figure 3.

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Figure 8 shows schematic drawing explaining how the

results from the stacking assays of figure 2 arise.

Figure 9 shows a graph of extent of denaturation plotted
against temperature for HIG and Sandoglobulin,
5   demonstrating how derivatisation leads to a greater
degree of denaturation at a given temperature.

Detailed Description
Production of Antibodies

10   The precursor antibodies may be obtained commercially or
prepared using techniques which are standard in the art.
Human precursor antibodies can be obtained as
commercially available polyclonal mixtures of antibodies
such as Sandoglobulin or CLB, or obtained from large
15   universal phage display libraries. Sandoglobulin is a
polyclonal IgG/glucose preparation that is produced and
commercially available from Sandoz. CLB is a polyclonal
IgG preparation which can be obtained from the Central
Laboratory of the Blood Transfusion Service of the
20   Netherlands Red Cross, Amsterdam, Netherlands.

Derivatised antibodies made from human precursor
antibodies have the advantage that they enable the use of
repeat treatments due to the absence of the human anti-
25   mouse antibody (HAMA) response (Schroff et al, Cancer
Research, 45:879-885, 1985; DeJager et al, Proc. Am.
Assoc. Cancer Res. 29:377, 1988). HAMA responses have a
range of effects, from neutralisation of the administered
antibody leading to a reduced therapeutic dose, through
30   to allergic responses, serum sickness and renal
impairment.

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In some instances, antibodies of other species may be
suitable precursor antibodies. Methods of producing such
antibodies are very well known in the art and include
immunising a mammal (e.g. mouse, rat, rabbit, horse,
5   goat, sheep or monkey) with the protein or a fragment
thereof. Antibodies may be obtained from immunised
animals using any of a variety of techniques known in the
art, and screened, preferably using binding of antibody
to antigen of interest. For instance, Western blotting
10   techniques or immunoprecipitation may be used (Armitage
et al, Nature, 357:80-82, 1992). Isolation of antibodies
and/or antibody-producing cells from an animal may be
accompanied by a step of sacrificing the animal.

15   In some embodiments, the precursor antibodies are
monoclonal. Monoclonal antibodies can be made using the
hybridoma method first described by Kohler et al, Nature
256:495, 1975, or made using recombinant means. Thus, an
antibody specific for a protein may be obtained from a
20   recombinantly produced library of expressed
immunoglobulin variable domains, e.g. using lambda
bacteriophage or filamentous bacteriophage which display
functional immunoglobulin binding domains on their
surfaces; for instance see WO92/01047. The library may
25   be naive, that is constructed from sequences obtained
from an organism which has not been immunised with any of
the proteins (or fragments), or may be one constructed
using sequences obtained from an organism which has been
exposed to the antigen of interest.
30   Monoclonal antibodies can be subjected to the techniques
of recombinant DNA technology to produce other antibodies

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or chimeric molecules which retain the specificity of the
original antibody. Such techniques may involve
introducing DNA encoding the immunoglobulin variable
region, or the complementarity determining regions

5   (CDRs), of an antibody to the constant regions, or
constant regions plus framework regions, of a different
immunoglobulin. See, for instance, EP 0184187 A, GB-A-
2188638 or EP 0239400 A. A hybridoma producing a
monoclonal antibody may be subject to genetic mutation or

10 other changes, which may or may not alter the binding
specificity of antibodies produced.

Pharmaceuticals

The antibodies of the invention can be formulated in
compositions for therapeutic or diagnostic use. These

15 compositions may comprise, in addition to one of the
above substances, a pharmaceutically acceptable
excipient, carrier, buffer, stabiliser or other materials
well known to those skilled in the art. Such materials

20 should be non-toxic and should not interfere with the
efficacy of the active ingredient. The precise nature of
the carrier or other material may depend on the route of
administration, e.g. oral, intravenous, cutaneous or
subcutaneous, nasal, intramuscular, intraperitoneal

25 routes, and can be readily selected by those skilled in
the art.

For intravenous, cutaneous or subcutaneous injection, or
injection at the site of affliction, the active

30 ingredient will be in the form of a parenterally
acceptable aqueous solution which is pyrogen-free and has

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suitable pH, isotonicity and stability. Those of
relevant skill in the art are well able to prepare
suitable solutions using, for example, isotonic vehicles
such as Sodium Chloride Injection, Ringer's Injection,
Lactated Ringer's Injection. Preservatives, stabilisers,
buffers, antioxidants and/or other additives may be
included, as required.

In therapeutic embodiments, the derivatised antibodies
are typically administered in an individual in a
"prophylactically effective amount" or a "therapeutically
effective amount" (as the case may be, although
prophylaxis may be considered therapy), this being
sufficient to show benefit to the individual. The actual
amount administered, and rate and time-course of
administration, will depend on the nature and severity of
what is being treated. Prescription of treatment, e.g.
decisions on dosage etc, is within the responsibility of
general practitioners and other medical doctors, and
typically takes account of the disorder to be treated,
the condition of the individual patient, the site of
delivery, the method of administration and other factors
known to practitioners. Examples of the techniques and
protocols mentioned above can be found in Remington's
Pharmaceutical Sciences, 16th edition, Oslo, A. (ed),
1980.

Alternatively, targeting properties of the antibodies may
be used to deliver the active agent more specifically to
locations in a patient's body at which immobilised IgG,
e.g. agalactosyl IgG, accumulates.

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As mentioned above, the antibodies of the invention can
be used to deliver one or more functional moieties to
locations associated with immobilised IgG, the functional
moiety being provided by the antibody itself or by the

5   conjugation of the antibody to another molecule. An
example of the former is the use of an antibody specific
for an inflammatory mediator, such as tumour necrosis
factor (TNFα). An example of the latter is conjugating
the antibody to a precursor form of a drug, for

10   conversion to the active form by an activating agent
produced in, or targeted to, the cells to be treated.
This type of approach is sometimes known as ADEPT which
involves targeting the activating agent to the cells by
conjugation to a cell-specific antibody, see for example

15   EP 0415731 A.

Materials and Methods

1.   Production of Monoclonal Antibody 3C4
An IgM monoclonal (3C4) with specificity towards terminal

20   N-acetylglucosamine (GlcNAc) was produced by the UCL
Monoclonal Antibody Unit by *in vitro* culture. Initial
screening of clones used terminal N-acetylglucosamine
rich ovalbumin and then agalactosyl IgG. The 3C4
monoclonal was purified from culture supernatant using

25   GlcNAc-sepharose®and eluted with 0.5M GlcNAc. It was
then dialysed and biotinylated. This antibody was used
as a probe for GlcNAc residues in the experiments
described below.

30   2.   3C4 Stacking Assays
(i)   Nunc®maxi-sorp plates were coated with 50μl of

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Protein A at 2.5mg/ml in PBS overnight at 4°C. Plates were washed with PBS/0.05%Tween® 20 (PT) and block d with 100µl of 1% BSA in PT (PBT) for 1h at 37°C. The plates were then washed with PT, and the human IgG to be tested
5 was added in 100mM glycine/150mM NaCl pH 8.2 buffer as serial five fold dilutions in triplicate starting at 20µg/well in 50µl (400µg/ml) for six dilutions such that the lowest amount was around 6ng/well. The plates were then incubated for 2h at 37°C, and then washed five times
10 with PT. 100µl of PBS was added to each well and the plates were then floated on a water bath at 85°C for 15 minutes to denature the IgG and expose the Fc N-linked oligosaccharides. The PBS was shaken out and 125ng of biotinylated 3C4 monoclonal was then added per well in
15 PBT and incubated overnight at 4°C. Following five further washes visualisation of the bound 3C4 was achieved by addition of 0.1mg 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulphonic acid] in 50µl 0.1M citrate phosphate, pH 4.1 containing 0.005% hydrogen peroxide,
20 and incubation in the dark for 10 minutes at room temperature. The reaction was stopped by the addition of 50µl of 0.2% sodium fluoride and absorbance measured at 650nm-490nm.

25 (ii) As a variation on the above assay, human IgG was also coated directly to Nunc® Maxi-sorp plates in 50µl PBS at the same serial dilutions 20µg/well to 6ng/well as stated above. Efficient coating was achieved after 2h at 37°C. From this point the assay was identical to the one
30 above from the heating step, i.e. 100µl of PBS was added to each well and the plates were then floated on a water

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bath at 85°C for 15 minutes. This assay gave higher
levels of bound human IgG and differs from the previous
assay with respect to the orientation of the initial IgG
monolayer.

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3.   Thiolation of Human IgG
Human IgG (30mg) was dissolved in 0.5ml of 15mM ammonium
bicarbonate, with all solutions being prepared and kept
oxygen free by using degassed solvent and being

10   maintained under nitrogen. 0.63mg of 2-iminothiolane-HCl
in 1 ml of 15mM ammonium bicarbonate was added and mixed
gently on a roller device for 20 minutes. The
derivatised IgG was then separated from the excess 2-
iminothiolane using a disposable Sephadex$^{@}$ G-25 (PD10)

15   column that had been blocked with 1% human serum albumin
in 0.9% NaCl, followed by equilibration in 15mM ammonium
bicarbonate. The elution of the derivatised human IgG
was achieved with 15mM ammonium bicarbonate and monitored
by 280nm absorbance. The tin/tartrate solution was made

20   as follows. Di-sodium tartrate (4mg/mg IgG) was
dissolved in 10ml of sterile oxygen free water. 4µg of
tin (II) chloride per mg IgG was added to the solution
(stock solution 750µg Sn^{2+}/mol in 1% HCl).

25   The IgG column eluate was added to the 10 ml solution of
tartrate and tin and brought to a final concentration of
1mg IgG/ml using the derivatisation buffer.

4.   Preparation of Concanavalin A Positive Human IgG
30   A 2ml bed volume column of Con A Sepharose$^{®}$ (Sigma) was
equilibrated with 50mM Tris pH 7.2 with 200mM sodium

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chloride and 2mM $MnCl_2$, $CaCl_2$. 10mg of human
immunoglobulin that had previously been dialysed and
freeze dried was dissolved in 1ml of the above buffer and
the applied to and then allowed to sit on the column for

5   10 minutes at room temperature. The column was then
washed with several column volumes of the above buffer
and the Con A unbound IgG fraction eluted and monitored
by 280nm absorbance. The Con A positive fraction was
then recovered by elution with 5 column volumes of 100 mM

10   HCl and then the pH was returned back to neutrality as
soon as possible with a minimal volume of a 1M Tris
solution. The absorbance of the bound fraction was
measured at 280nm, it was usually found that
approximately 25% of the IgG was Con A positive. Both

15   fractions were dialysed against water and freeze dried
prior to further analysis.

Results and Discussion

Figure 1, top, shows that the anti-GlcNAc reactivity of
derivatised IgG (HIG) as described in method (3) is

20   greater than the starting material (Sandoglobulin) at any
fixed concentration. Since both the Sandoglobulin and
the HIG have the same chemical content of terminal N-
acetyglucosamine residues on the carbohydrate chains

25   attached to the IgG molecules, respectively, the increase
in the reactivity must result from an increase in
accessibility of the carbohydrate residues to the 3C4
anti-GlcNAc monoclonal probe.

30   The carbohydrate residues on the Fc fragment IgG are
normally inaccessible since they are buried inside the

protein (see Figure 7). By heating to 85°C, some of the
carbohydrates chains become accessible and can then be
detected by 3C4. The increase in accessible carbohydrate
chains for HIG, when compared to Sandoglobulin, suggests

5   that at 85°C the HIG is more easily thermally denatured,
exposing a greater number of chains. This is shown in
Figure 9, which shows a graph showing how the
accessibility of GlcNAc residues of HIG and the
Sandoglobulin starting material varies with temperature.

10   This demonstrates that the derivatisation reaction
reduces the temperature at which the carbohydrate chains
become available towards physiological temperature (see
Figure 1, bottom). This shift is particularly
advantageous as it provides a method of derivatising the

15   antibodies while avoiding loss of the antibody binding
activity caused by thermal denaturation, e.g. making it
possible to make derivatised antibodies that retain a
useful activity associated with the antibody binding
reaction, such as binding specificity for an inflammatory

20   mediator. The use of thermal denaturation to "flip out"
the sugars is not a useful method since (1) on returning
to physiological temperatures the sugars will flip back
in and (2) the thermal denaturation may disrupt the
antigen binding site on the antibody. Accordingly, the

25   two step method for producing derivatised antibodies
described in this embodiment provides (1) a chemical
denaturant to "flip out" the sugar chains at
physiological temperatures followed by (2) a chemical
derivatisation to prevent them from flipping back.

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When adding increasing amounts of IgG to a microtitre

well saturation would be expected. Figure 2 shows that whereas Sandoglobulin saturates, there is a continual increase in anti-GlcNAc reactivity of the HIG as the concentration of HIG applied to the microtitre plates is

5   increased. This indicates that a new top surface monolayer is being formed for the case of HIG which contains increasing proportions of anti-GlcNAc reactive IgG. This "stacking" of anti-GlcNAc reactive IgG onto the initial Protein A immobilised monolayer and

10   subsequent monolayers is a property unique to the HIG and not found for Sandoglobulin. This phenomenon is described in Figure 8, which show how the proportion of derivatised antibody (shown by the filled circles), as compared to the immobilised IgG (shown by the open

15   circles), increases in successive layers of the stack.

Figure 2 demonstrates that the stacking of anti-GlcNAc reactive IgG also occurs when the initial monolayer is not oriented by the coating of the plate (i.e. Protein

20   A). Figure 2 also demonstrates that even in the absence of heating there is a significant amount of anti-GlcNAc reactive IgG in the HIG preparation and that essentially none exists in the Sandoglobulin.

25   IgG is also known to contain carbohydrate residues in the Fab region of the molecule. The Fab carbohydrates are accessible to lectin binding and therefore IgG molecules containing Fab associated carbohydrates can be separated by affinity chromatography. Figure 3 (bottom) shows that

30   the percentage of Con A bound (IgG containing Fab carbohydrates) and Con A unbound (IgG with no Fab

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carbohydrates) is the same for Sandoglobulin and HIG. Figure 3, top, however shows that all of the "stacking" IgG is found in the Con A bound fraction of HIG. These species are shown schematically in Figure 7. Figure 3 also suggests that in cases in which not all of the derivatised antibodies are capable of stacking, a separation step should be employed to separate the stacking antibodies.

These results suggest that the "stacking" IgG has three specific characteristics:

(a) Firstly, "stacking" HIG is reactive with anti-GlcNAc monoclonal antibodies which are probing the carbohydrates on the Fc fragment.

(b) Secondly, it is only those HIG molecules which also have a Fab associated carbohydrate which stack.

(c) Thirdly, the increased proportion of anti-GlcNAc reactive material which is found when the HIG concentration is increased indicates that the "stacking" IgG prefers to stack itself (i.e. self-association).

In patients with rheumatoid arthritis, there is an increase in the serum content of agalactosyl IgG glycoform. This glycoform is disease associated and is characterised by having an increased proportion of its Fc-associated carbohydrate chains terminating with N-acetylglucosamine rather than galactose or sialic acid, see Figure 6(b). This form of IgG therefore has an

increased anti-GlcNAc reactivity with the 3C4 monoclonal
similar to the *in vitro* produced HIG. Immobilised
agalactosyl IgG will therefore act as a preferential
surface for the "stacking" of HIG *in vivo*. In this way,
HIG is able to selectively bind to tissue surfaces (e.g.
synovial joints) of patients with autoimmune disorders in
the presence of large concentrations of endogenous IgG.

Figures 4 and 5 demonstrate that the iminothiolane
derivatisation reaction itself is not sufficient to
produce HIG which is able to stack. This result is
unexpected and novel. The most probable mechanism is
that certain buffers (e.g. ammonium bicarbonate) are able
to briefly cause the carbohydrate residues to "flip out",
exposing cryptic amino acid residues which can then be
derivatised with the iminothiolane. Once derivatised,
the presence of the iminothiolane groups sterically
prevent the carbohydrate chains from flipping back inside
the protein, see Figure 7(b). An alternative mechanism
is that when the carbohydrate chains are "flipped out",
the iminothiolane derivatisation of amino acid residues
remote from the carbohydrate binding site results in
stabilisation of a protein conformation in which the
carbohydrate chains spend more time "flipped out" at 37°C
(see Figure 1(b)). That is, the dynamic equilibrium is
shifted such that the sugar chains become more mobile at
body temperature and consequently the protein surface
becomes exposed for a greater time for interaction with
carbohydrate chains from adjacent molecules.

Figure 6(a) shows how the derivatised antibodies of the

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invention bind to anti-type II collagen IgG antibodies
that are found immobilised in the joint of RA patients.
The derivatised antibodies have flipped out carbohydrate
residues that are available to bind to the anti-type II

5   collagen IgG antibodies, and have binding sites opened up
for binding by the agalactosyl carbohydrate chains of the
immobilised IgG. This means that the derivatised
antibodies can bind to sites where the immobilised
antibodies are located and stack to provide amplification

10   of a label for diagnosis or another kind of effector
moiety for a therapeutic purpose.

Figure 6(b) shows the difference in the structure of
carbohydrate chains in agalactosyl and normal antibodies,

15   the agalactosyl IgG missing galactosyl and sialic acid
residues from the terminus of the carbohydrate chain.

Figure 7 shows a schematic drawing of the species
involved in the experiments investigating the stacking

20   properties of the HIG derivatised antibodies described
above. Thus, the derivatisation reaction flips out the
Fc carbohydrates (compare Figure 7 Con A bound and Figure
7 (HIG)) that were bound to the internal protein surface.
This surface (binding site) is now vacant and can

25   interact with another carbohydrate chain on an adjacent
IgG molecule. The data in Figure 3 indicates that this
carbohydrate chain is the one attached to the Fab (see
Figure 7 Con A bound) of an adjacent IgG molecule. Serum
agalactosyl IgG (Figure 6(b)) behaves like the

30   derivatised antibodies since it is missing carbohydrate
residues exposing the same protein surface which occurs

in derivatised IgG when the carbohydrate chains flip out.

The references mentioned herein are all expressly incorporated by reference.

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